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FINAL TECHNICAL REPORT

STERILIZATION ACTION OF CHLORINE AND IODINE
ON BACTERIA AND VIRUSES IN WATER SYSTEMS

1 July 1962 to 30 June 1966

by

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Final Report to the Commission on Environmental Hygiene
of the Armed Forces Epidemiological Board

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ABSTRACT

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Final Report to the Commission on Environmental Hygiene of the Armed Forces Epidemiological Board

Since neither infectious RNA nor DNA is affected, iodine as a disinfectant probably inactivates bacteria and viruses by the iodination of the appropriate protein component.

The bactericidal action of iodine is complete within one minute of contact at 37°C. The rate of inactivation is not materially reduced by high concentrations of iodide ion. The destruction of bacteria continues until all iodine is consumed or, in case of residual iodine, until all bacteria are destroyed. The minimum number of iodine molecules required to destroy one bacterium varies with the species. For H. influenzae it was calculated to be 1.5×10^4 molecules of iodine per cell. When bacteria are treated with iodine, the inorganic phosphate up-take and oxygen consumption by the cells immediately ceases. Bacterial cells reacted with radioiodine show very little in the cell fraction, indicating that most likely an oxidation of -SH groups rather than a substitution into tyrosyl moieties occurs.

The inactivation of poliovirus and f₂ RNA bacteriophage by iodine is considerably inhibited by low levels of iodide ion concentration. At pH 6.0 or less iodine inactivation of f₂ phage was incomplete. The rate of inactivation by iodine in the presence of iodide ion for the f₂ virus and its host E. coli is different. The difference most likely is due to the nature of the iodine sensitive proteins involved.

The virucidal properties of iodine in waters of low pH and high organic content could be inadequate regardless of the free iodine concentration when virus or viruses involved require iodination of tyrosine for inactivation.

At the existing stage of the investigation on the mode of action of bromine on virus and cells, further work will be required to form definite conclusions. A general tentative summary is as follows:

- (1) Bacterial cells appear to be more resistant to bromine than the phage.
- (2) Bromine activity on cells and phage varies directly with the hydrogen ion concentration and temperature.
- (3) The presence of the bromide ion enhances phage and inhibits cell inactivation by bromine.
- (4) The activity of RNA harvested from bromine treated phage is less affected than the intact phage.

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Final Technical Report

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Sterilization Action of Chlorine and Iodine on Bacteria and Viruses in Water Systems

GENERAL STATEMENT OF THE PROBLEM

The problem is one of providing a safe drinking water for the individual soldier in the field under a wide variety of existing physio-chemical conditions of water and pathogen burden. Under most conditions, simple chemical disinfection with a suitable halogen has satisfactorily solved the problem. However, under worst raw water conditions often encountered, the effectiveness of treatment by halogens alone is somewhat in doubt, especially in regard to viruses for which the mode of inactivation may be different from that of bacterial pathogens or parasitic cysts. The unusual pattern of hepatitis-gastroenteritis morbidity reported from troops demand further investigation on the behavior of viruses to disinfecting agents before rejecting the possibility of water-borne hepatitis.

INTRODUCTION

Chlorine and its compounds has been used as a water disinfectant for more than 50 years, and much has been published on the possible chemical species, free and combined, and their kinetics of disinfection at various concentrations, times of contact, pH and temperature. Nevertheless, the nature and mode of action of the chlorine species is still not definitely known. The requirement of a 30-minute "free chlorine" residual at pH 7.0 of 2.5 ppm at 22° to 25°C, and 10 ppm at 2° to 5°C for destroying the cysts of E. histolytica led to the development of iodine tablets (Globaline) for more rapid and effective inactivation of this parasite in field water supplies. The tablet, containing tetraglycine hydroperiodide, is more stable than the organic chloramines and will release 8 mg of I₂ when dissolved in water. According to Morris et al.(1), sodium acid pyrophosphate was employed as an active ingredient in the Globaline tablet to impart desirable physical properties to the tablet, and its acidity serves to lower the pH of the natural waters and thus prevent hydrolysis of the elemental iodine into less germicidal substances. Chang and Morris (2) have thoroughly demonstrated the disinfecting efficiency of iodine on the enteric bacteria including S. typhosa and V. cholerea. Against the cysts of E. histolytica iodine was superior to chlorine and, in general, the tablet could be considered a "universal" water disinfectant for a wide range of water quality, temperature and pH.

Comparison of the virucidal efficiency of iodine and chlorine is, at present, inconclusive from existing experimental data. There is the

recent report that a commercial organic iodine did not completely inactivate poliovirus in the presence of organic substances (3). Also, the Tobacco Mosaic Virus is resistant to iodine in high concentrations of iodide (4,5). In view of the limited information concerning the mode of bactericidal and virucidal action of iodine it was decided to inaugurate study with this halogen.

METHODS AND MATERIALS

1. Bacteria

Strains: Haemophilus influenzae, E. coli K-12 and Salmonella abortusovine (ATCC9842) were used as test strains. H. influenzae was cultivated in brain heart infusion (Difco) with hemin and diphosphopyridin nucleotide (DPN). The test bacteria were washed two times with 0.14 M saline and the washed bacteria were resuspended in 0.1 M phosphate buffer to maintain the test system at pH 6.0. Cultures so prepared were mixed with iodine solutions of various predetermined concentrations containing 1 to 1.2 molar ratio of iodine to iodide and maintained at 37°C in a constant temperature water bath shaker. The iodine reaction was terminated at desired intervals of time by pouring treated cultures into 10 to 100 fold volumes of 10⁻³ M solution of sodium thiosulfate.

Halogen concentrations were measured by both iodometric and amperometric titration methods described in Standard Methods (6). Bacterial DNA and RNA were separated by the Schmidt-Thanhauser Method modified by Hershey (7).

Carrier-free I¹³¹ iodide was converted to free iodine by the addition of a known excess of hypochlorous acid. This was followed by adding an excess of KI to destroy the residual hypochlorous acid leaving a stoichiometric equivalent of free iodine. A solution of hypochlorous acid was prepared by bubbling chlorine gas into 0.1 M phosphate buffer solution (pH 6). The resulting solution can be stored in the dark in a cold room for several months without significant decrease in the concentration.

2. RNA Bacteriophage

A bacteriophage strain f₂ containing RNA was kindly supplied by Dr. N. D. Zinder through Dr. T. Fukasawa. The f₂ phage was prepared in essentially the same way as described by Loeb and Zinder (8). The phage stock solution, containing approximately 5 x 10¹² PFU/ml, was diluted to 10⁻⁴ in 0.05 M phosphate buffer, pH 7, containing 0.14 M NaCl (PBS), and used as a test phage.

Para-chloromercuribenzoic acid, obtained from the Sigma Chemical Company, Inc., was diluted in 0.05 M tris-HCl buffer, pH 8, containing 0.14 M NaCl.

3. Poliovirus

Poliovirus type 1 of Connaught Medical Research Laboratory, University of Toronto, was kindly supplied by Dr. N. Nathanson. Prior to experimental use, the virus was plaque-purified twice on Clonal HeLa S-3 cells (9).

Virus stock was prepared by inoculating on FL cells (10), purchased from Microbiological Associates, Bethesda, Maryland, and stored at -65°C . The test poliovirus was prepared by diluting it into 100 times its volume of buffer saline (PBS) according to the method described by Dulbecco.

Tissue culture media: HeLa S-3 and FL cells were grown on Medium 199 with 10 per cent calf serum and 100 units/ml of penicillin, 100 $\mu\text{g}/\text{ml}$ of streptomycin and 20 units/ml of mycostatin.

Plaque assay (11): Poliovirus titrations were carried out by determination of plaque-forming units (PFU) with 55-mm plastic dish technique.

Poliovirus RNA: The procedure used for concentrating poliovirus was essentially similar to that described by Levintow and Darnell (12). Two thousand ml of poliovirus culture of FL cells, having 10^8 PFU/ml, was centrifuged to remove cellular debris at 10,000 r.p.m. for 60 minutes. The supernatant fluid was concentrated to one-tenth the original volume under reduced pressure in a rotary evaporator at 32° to 35°C , with a few drops of octyl alcohol as antifoaming agent. The precipitated salts and cellular material resulting from evaporation were eliminated by centrifugation at 2500 r.p.m. for 20 minutes. The supernatant fluid was then centrifuged at 40,000 r.p.m. for 3 hours. The resulting pellet was resuspended in 10 ml of 0.02 M PBS (pH 7.2) with the aid of glass tissue homogenizer. The infectious RNA extraction was essentially similar to that described by Paranchych (13).

Assay of infectious RNA: FL cells were washed with Hank's balanced salt solution and then treated with 4 ml of 0.9 M NaCl solution in 0.04 M phosphate buffer (pH 7.2) (14) for 5 minutes at room temperature. The washing solution was removed and cells were infected for 15 minutes at room temperature with 0.1 ml of the RNA which was diluted previously with 0.04 M phosphate buffer (pH 7.4) containing 1.2 M NaCl. The usual agar overlay was added without removing the inoculum.

EXPERIMENTS

1. Iodine Demand of Bacteria and Minimal Bactericidal Unit of Iodine

Although ten minutes was initially taken as a tentative reaction time for iodine with bacteria, it was found that the killing effect of iodine for 3×10^8 cells/ml of Haemophilus influenzae was complete within one minute at room temperature. Thereafter, the survival number of bacteria remained constant (Figure 1).

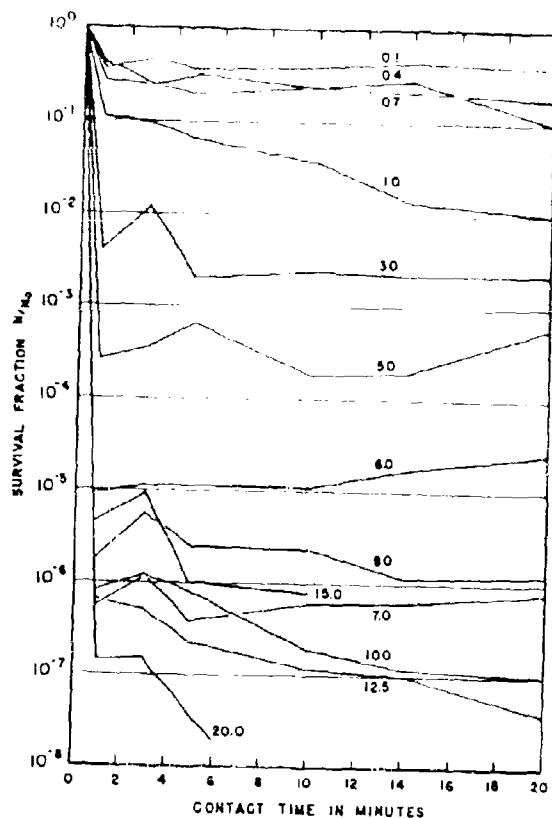


Figure 1. Survival fraction of *Haemophilus influenzae* in varying concentration of iodine against contact time. pH 6; temperature, 37°C

The logarithm of the bacteria survival fraction (N/N_0) plotted against iodine concentration revealed an almost linear relationship (Figure 2). Calculation of the minimum number of iodine molecules required to kill one cell of *Haemophilus* may be made at the point at which 37 per cent survival is observed. An I_2 concentration of 2 μ moles/ml (0.5 mg/l) was required for 3×10^8 cells per milliliter or 1.6×10^4 molecules of iodine per cell.

Efforts to demonstrate the development of bacteria resistant to iodine by selection of large population number and retesting survivors was entirely unproductive.

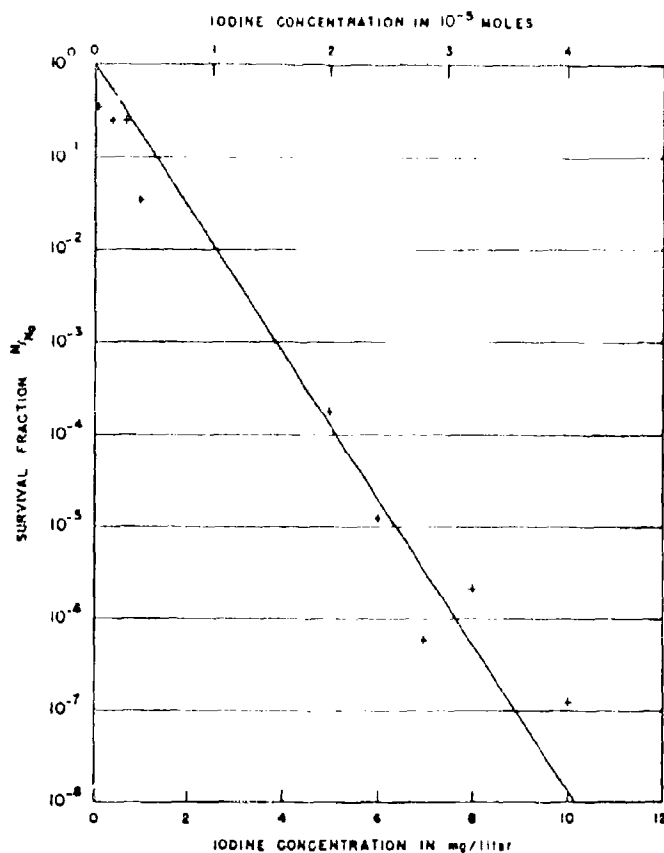


Figure 2. Fraction of *H. influenzae* surviving after 10 minutes exposure to varying concentrations of iodine. pH 6.0; temperature 37°C

2. Bacterial Metabolism after Contact with Iodine

The oxygen consumption of iodine treated *Salmonella abortusovine* cells was measured after tipping various concentrations of iodine from the side arm of the Warburg manometer. The results of these experiments are given in Figure 3 and oxygen consumption ceased immediately upon addition of iodine sufficient to cause 99 per cent killing effect. In lower concentrations of iodine, the amount of oxygen taken up was proportional to the number of cells surviving.

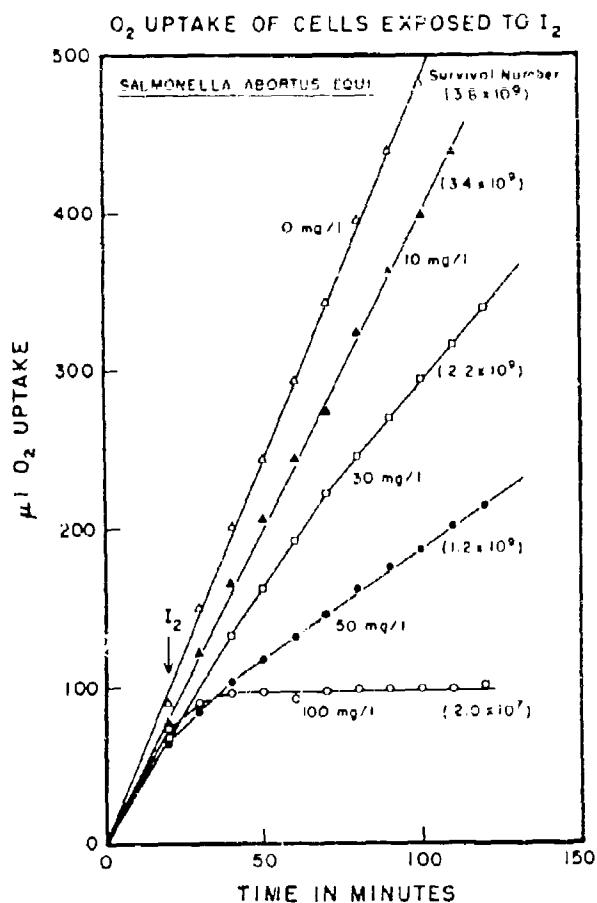


Figure 3. Oxygen uptake of *Salmonella abortus equi* after treatment with various concentrations of iodine at pH 7.0 Temperature, 37°C.

The effect of a similar iodine treatment on the ability of the cell to incorporate inorganic phosphate in synthesis of RNA and DNA was tested. It can be seen in Figure 4 that the P^{32} up-take ceased immediately after contact with iodine, and there was no increase in P^{32} incorporation into RNA and DNA with passing time in the iodine treated cells, whereas the untreated control incorporated P^{32} into both RNA and DNA fractions.

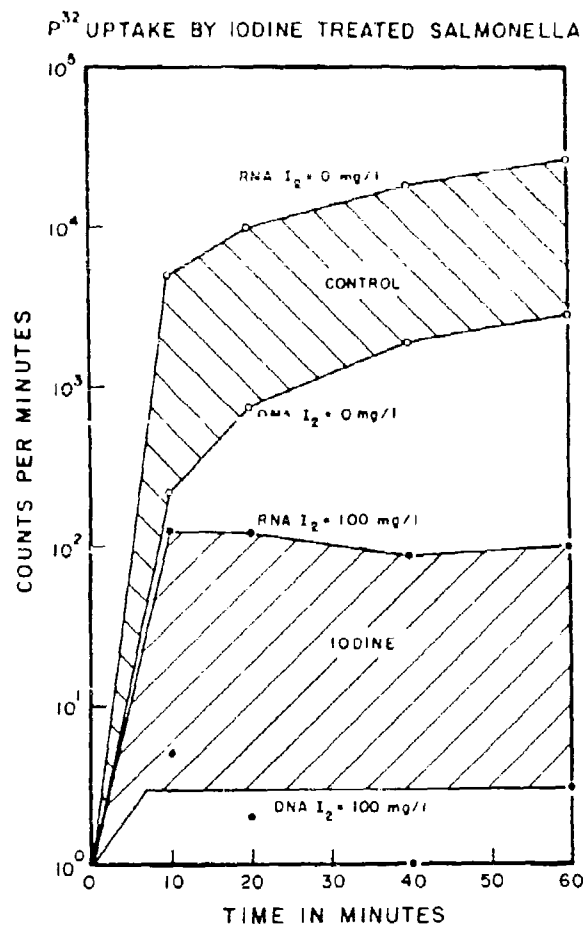


Figure 4. The effect of iodine on the ability of *S. abortus equi* to utilize inorganic phosphorus in synthesis of RNA and DNA. Temperature, 37°C; pH 7.0.

In an attempt to determine whether the iodine demanded for bacterial destruction involves an oxidation of the -SH groups or substitution into the tyrosine and histidine, an experiment employing radioiodine was performed. The cells of *Salmonella abortus equi* were reacted for 15 minutes with I_2^{131} , washed with PBS, centrifuged, and the cells and supernatant counted separately for radioiodine. Very little radioiodine was found in the fraction, while 99 per cent of the radioactivity was measured in the supernatant. From this it may be inferred that very little of the iodine was incorporated into the bacterial component. The bactericidal action of iodine was not reversed by reducing agents such as glutathione, DPNH and cysteine.

3. Action of Halogen on Transforming Deoxyribonucleic Acid (DNA)

Purified transforming DNA containing less than 1 per cent of protein was extracted from strains of *Haemophilus influenzae* resistant to streptomycin (S^r), cathomycin (C^r), erythromycin (E^r) and kanamycin (K^r), and, in addition, a strain which utilized protoporphyrin (PP^+) in the dark in place of hemin was used. The DNA was dissolved in 0.1 M phosphate buffer saline (PBS), and mixed with the halogen, chlorine, bromine, and iodine under different conditions of time pH, temperature, and concentration. The reaction with the halogens was stopped by the addition of sodium thiosulfate to the various samples. The residual transforming activity in these samples was determined by incubating with competent *H. influenzae*, and then plating on agar containing individual antibiotics or with protoporphyrin instead of hemin and cultured in the dark.

Summarized in Figure 5 are the results of a series of experiments in which the number of transformations per 0.01 μ g of transforming DNA remaining after reaction with the different halogens is plotted against the initial halogen concentration.

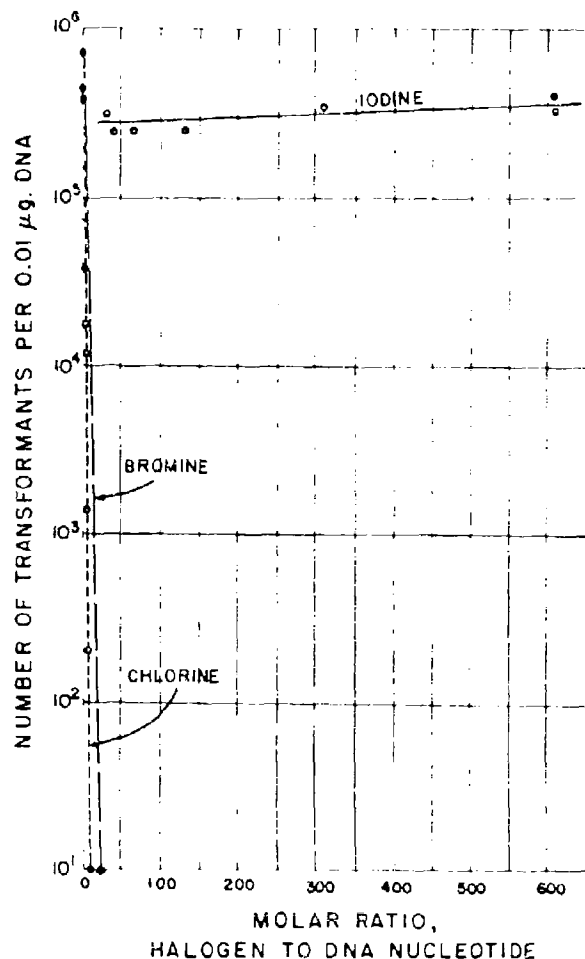


Figure 5. Effect of iodine, chlorine and bromine on transforming DNA

It is clear from these results that the transforming activity was destroyed by chlorine and bromine but not by iodine. Even after 4 hours of contact at 37°C with 2 mM (500 mg/l) of iodine in 2.4 mM KI, all five genetic markers (S^r , C^r , E^r , K^r and PP) were almost fully active. In some experiments DNA survived reaction with iodine at 45°C and pH 8.5. Heat denatured DNA was also resistant to iodine. Dialysis of DNA after treatment with I^{131} left no detectable iodine bound to the nucleic acid, so that there could not have been an addition or substitution reaction of iodine. The iodine failed to change ultra-violet absorption of the nucleic acid in contrast to a pronounced leveling of the peak by chlorine.

Thus, despite its powerful bactericidal action, iodine is unable to inactivate bacterial DNA and indicates that bactericidal action of iodine is limited to the protein capsule.

4. Infectious RNA from Iodinated Phage

Partially purified f2 phage, having 1×10^{13} plaque forming units per ml (PFU) was treated with 0.2 mM of iodine containing 0.24 mM of KI for 20 minutes at 27°C. The fraction of phage surviving was 1.8×10^{-5} or .0018 per cent.

After reducing the residual iodine with sodium thiosulfate, the RNA was extracted and compared to that extracted from un-iodinated phage. Despite the apparent phage inactivation in the test, the RNA extract from the test and its control were found to be the same, as shown in Table I.

TABLE I
IODINATION OF INTACT PHAGE AND INFECTIVITY OF ITS RNA

	Infectivity in PFU/ml			Infectious RNA Found
	Before	After	Survival %	
Iodinated Phage	10×10^{12}	1.8×10^8	.0018	3.25×10^6
Control Phage	10×10^{12}	10×10^{12} *	100	3.65×10^6

*Control treated with thiosulfate only

Such a result suggests that either the protein coat of the phage is so compact that the iodine cannot reach the RNA or that the RNA is insensitive to iodine.

5. Resistance of Infectious RNA to Iodine

To test the sensitivity of infectious RNA to iodine, 2.6×10^6 PFU/ml of f₂ phage RNA was treated with 0.4 mM iodine containing 0.48 mM KI for 10 minutes at 27°C. Residual iodine persisted throughout the reaction until reduced by the addition of sodium thiosulfate. As shown in Table II the infectious RNA activity was completely resistant to the treatment of iodine.

TABLE II
RESISTANCE OF INFECTIOUS f₂ RNA TO IODINE

	Infectivity PFU/ml
Iodinated RNA	2.42×10^6
Control RNA	2.55×10^6

From these results it is quite clear that the inactivation of phage by iodine in minimal amounts of iodide may be attributed to its action on the protein coat of the phage. The action of iodine on the protein components is well known, and includes the oxidation of sulfhydryl (-SH) groups or substitution into tyrosyl or histidyl components (15). Since f₂ has no histidine residue in its protein coat (16), the reaction of iodine must be with the -SH and tyrosyl groups, although tryptophanyl residues could also be involved.

6. Poliovirus RNA Resistant to Iodine

The poliovirus RNA was exposed to 0.8 mM (200 mg/l) of iodine containing 0.96 mM of iodide at 25°C. At the indicated time the residual iodine was reduced by sodium thiosulfate. The survival infectivity of polio RNA was assayed by the methods as described. As can be seen in the following table, there is no indication of inactivation by iodine even for an exposure to iodine for 60 minutes.

Exposure time to iodine in minutes	Survivals (PFU/ml)
0	4.0×10^4
10	3.5×10^4
30	4.5×10^4
60	5.0×10^4

7. Iodide Inhibition of Iodination on Bacteria and f₂ Phage

The inactivation of both E. coli and the f₂ phage was complete at room temperature within 30 seconds with a 0.04 mM (10 mg/l) concentration of iodine in the presence of 0.048 mM KI (Figure 6a). It was observed, however, that small increases in KI concentration reduced the rate of virus inactivation but did not reduce the inactivation of E. coli. Even at 0°C, in the presence of 0.5 M KI, E. coli was inactivated within five minutes, where the f₂ virus was almost fully active after one hour of contact. These results are shown in Figure 6b, and suggest that, under conditions in which only the -SH groups are affected, the f₂ phage survived indicating either the absence or inaccessibility of the -SH in the protein coat or that the -SH groups are not essential in phage infectivity and multiplication.

Evidence to support this reasoning was obtained by reacting E. coli and f₂ phage with p-chloromercuribenzoate (PCMB), a -SH attacking agent. The survival of phage and E. coli obtained after the application of 10⁻³M PCMB is shown in Figure 6c. The striking similarity of inactivation rates obtained with PCMB and iodine inactivation in the presence of 0.5 M KI (Figure 6b) should be noted.

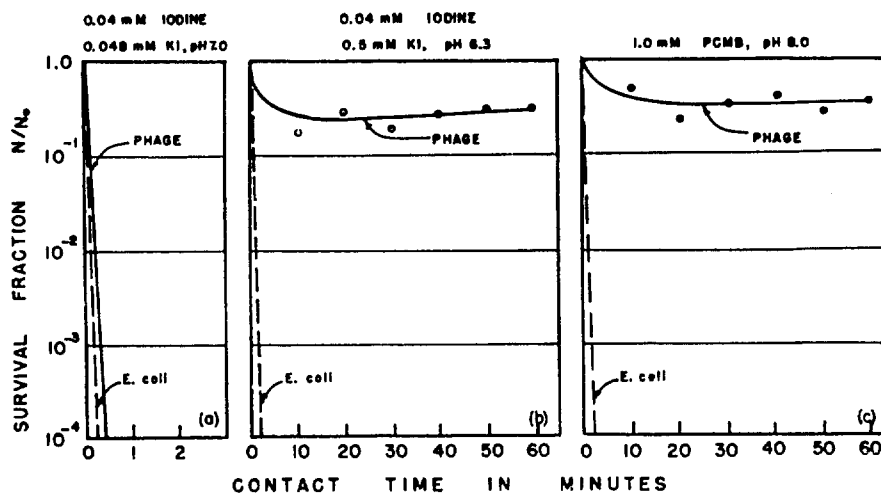


Figure 6. Survival of E. coli and f₂ bacteriophage reacted with 0.04 mM iodine containing (a) 0.048 mM KI at 37°C, (b) 0.5 mM KI at 0°C, and (c) compared to the survival obtained with 1 mM of sulphydryl reacting agents, PCMB.

8. Effect of KI Concentration on the Rate of Poliovirus and f₂ Phage

Inactivation by Iodine

Poliovirus has been reported to be resistant to PCMB (17,18) and it might be assumed that poliovirus could be resistant to iodine in the presence of KI. This assumption was tested as follows: 1.8 ml. of poliovirus suspension in PBS, having 1.6×10^6 PFU/ml, was treated for 20 minutes with 0.08 mM of I₂ which contained various concentrations of KI at room temperature. The reaction was terminated by adding one drop of sodium thiosulfate into the reaction mixture. The survival virus was assayed on FL monolayer cells by the plaque technique.

The f₂ phage was similarly assayed after 10 minutes of contact at room temperature with 0.04 mM iodine containing varying concentration of KI in 0.05 M PBS.

The inhibitory effect of KI concentration on iodine inactivation of poliovirus and phage is shown in Figure 7. As the concentration of iodide is increased the survival of polio and bacterial virus increases. When the KI concentration approaches 10^{-2} M inactivation is limited to less than 90 per cent. Below a concentration of about 10^{-2} M KI, it can be stated that the inactivation of virus varies inversely with approximately the cube of the iodide concentration. A 10-fold increase in iodine dosage with contact time prolonged to hours did not increase the inactivation of virus in concentrations of KI above 10^{-2} M.

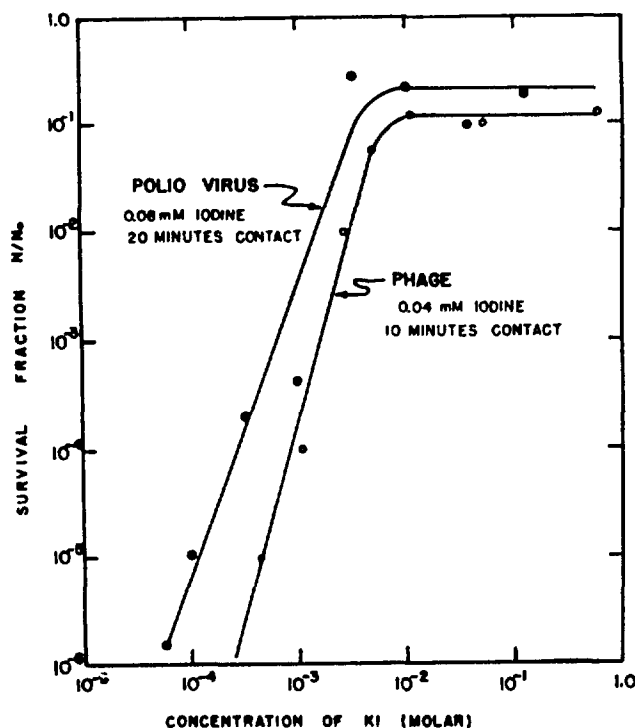


Figure 7. The effect of KI concentration of iodine inactivation of poliovirus type 1 and f₂ bacteriophage. Phage treated for 10 minutes with 0.04 mM iodine. Poliovirus treated for 20 minutes with 0.08 mM iodine. pH 7.0; temperature, 26°C.

Figure 8 shows the inactivation rate of poliovirus as a function of time with a constant 0.08 mM iodine at room temperature in varying concentrations of KI. It can be seen that with increasing concentrations of KI both the rate and degree of poliovirus inactivation is sharply decreased.

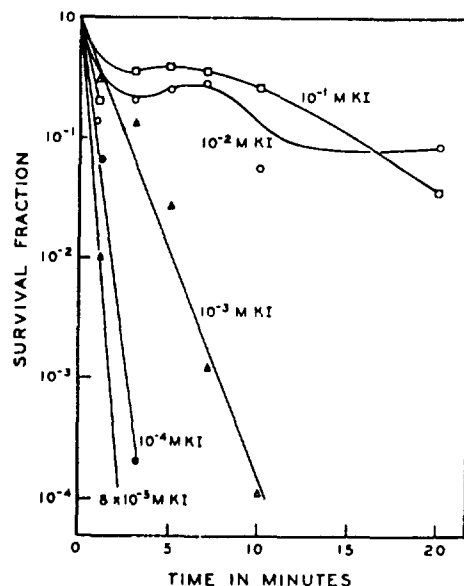


Figure 8. Kinetics of iodine inactivation of poliovirus type 1 in varying concentrations of KI. Poliovirus exposed for 20 minutes to 0.08 mM iodine at 26°C and pH 7.0.

9. Comparison of Iodination of Tyrosine to Iodine Inactivation of Phage

Tyrosine is the amino-acid residue most likely to be attacked by iodine at neutral pH and room temperature. In examining the action of iodine on tyrosine, Li (19) noted that the iodide ion retards the reaction. In this experiment the iodination of tyrosine was performed under conditions comparable to those used for the inactivation of phage.

L-Tyrosine (Calbiochem) was dissolved in pH 7.0, 0.05 M phosphate buffer, containing 0.14 M sodium chloride (PBS). Iodotyrosine was determined from the absorbency at 312 mμ wave length.

The results given in Figure 9a show that the iodination of tyrosine was very rapid in 0.6 mM KI with 0.5 mM of iodine, whereas in 100 mM KI and the same iodine concentration only 5 per cent of tyrosine was iodinated. Thus, the conditions which inhibit iodination of tyrosine (Figure 9a) also inhibits the inactivation of phage by iodine as shown in Figure 9b.

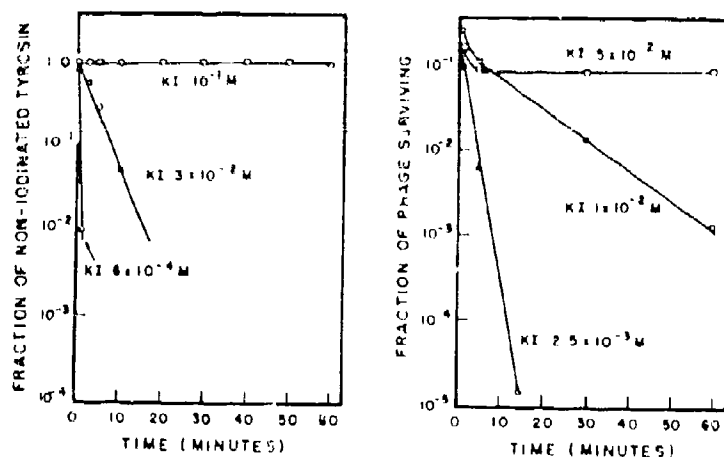


Figure 9a. Effect of varying amounts of KI on iodination of tyrosine with 100 mM iodine at pH 7.0 and $26^{\circ}C$.

Figure 9b. Effect of KI concentration on rate of phage inactivation with 0.04 mM of iodine at pH 7.0 and $26^{\circ}C$.

10. Effect of pH on Phage Iodination

Figure 10 shows the survival fraction of phage after contact for 10 minutes with 0.04 mM iodine in the presence of 0.048 mM KI at varying pH and at $26^{\circ}C$. The survival of virus increased with the hydrogen ion concentration below pH 6, or perhaps more important, the rate of inactivation increased as the square of the hydroxyl ion concentration. The reduction in rate of iodination of tyrosine with an increase in hydrogen ion concentration was noted earlier (19).

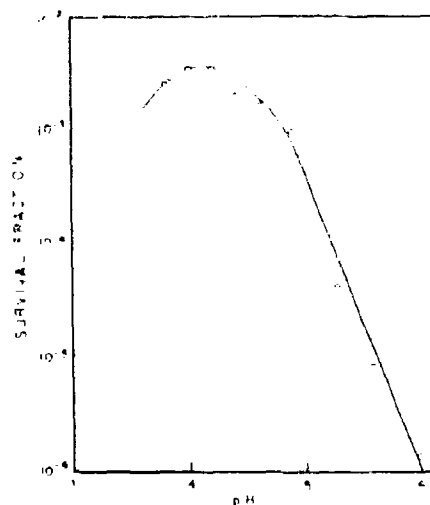


Figure 10. The effect of pH on the survival fraction of f₂ phage after 10 minutes contact at 26°C with 0.04 mM iodine containing 0.043 mM KI.

Additional experiments were performed to test the effect of pH on the rate of phage inactivation by iodine at the extreme temperatures of 5°C and 30°C. The initial inoculum was 8×10^{10} PFU/ml and survival was determined after treatment with 0.04 mM iodine (10 mg/l) at various periods of contact time up to 40 minutes. At both 5°C and 30°C the survival of phage following iodination increased as the hydrogen ion concentration increased. The effect was greatly intensified in water at 5°C, where at pH 4 to 5 more than one per cent of the phage survived up to 40 minutes of contact. At 5°C and pH 10, however, more than 7 logs of phage were inactivated in 30 seconds (99.9999% destruction).

Where inactivation of the phage is affected by pH, the kinetic curve appears to be in two stages: the first, an almost instantaneous inactivation of 1 or 2 logs, and the second, a much slower rate of inactivation.

From these data, an attempt was made to determine the inactivation rate constants as a function of pH at 30°C and 5°C. The constants determined at each value of pH have been plotted on Figure 11.

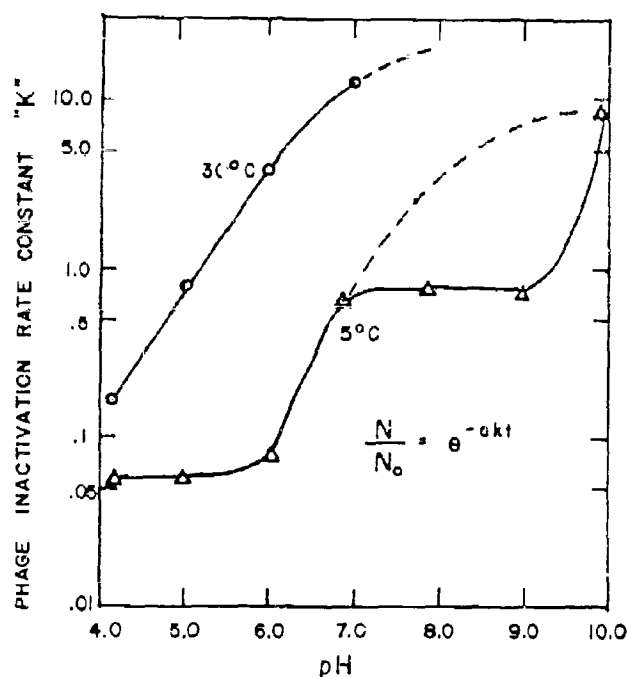


Figure 11. The inactivation rate constant, K , as a function of pH on phage treated with 0.04 mM iodine and 0.048 mM KI in varying time at 30° and 5°C.

The leveling of the rate between pH values of 8 and 9 on the 5°C curve was observed but could be due to a poor selection of buffer (tris amino-ethane) and its reaction with iodine. These two points should be reconfirmed using other buffers.

The effect of adding the Globaline tablets to 1000 ml of tap and distilled water was done to show the reduction in pH by the dehydrogen disodium pyrophosphate.

Number of Globaline Tablets Added	Hydrogen ion concentration	
	Tap Water	Distilled
0	7.5	5.7
1	6.1	4.5
2	5.8	4.2
4	5.5	4.0
8	5.1	3.8
16	-	3.7

The maximum recommended iodine tablet dosage has usually been one tablet per quart and 2 tablets per quart of turbid-colored water.

PRELIMINARY OBSERVATIONS WITH BROMINE

1. Effect of Bromine Concentration and Time of Contact on the Inactivation of Cells and Phage

A bromine concentration of 2 to 4 ppm was found to give a three to five log inactivation of cells and phage in 10 minutes at pH 7.5 and 0°C. The degree of inactivation was proportional to the residual bromine at the end of the contact period. Increasing the bromine dosage above 4 ppm did not materially alter the survival curve for cells and phage at the 10 minute contact time. A 4 ppm dosage will give, in 10 minutes, a 3 log cell reduction from an initial concentration of 5×10^8 cells/ml. Phage, on the other hand, for the same 4 ppm bromine dosage and 10 minute contact time will give about a 4 log reduction with an initial of 2×10^9 PFU/ml. It is difficult at this time to determine whether *E. coli* is more resistant to bromine than phage or whether the initial lag (a delay of about 5 minutes) is responsible for the difference in inactivation rate (see Figure 12).

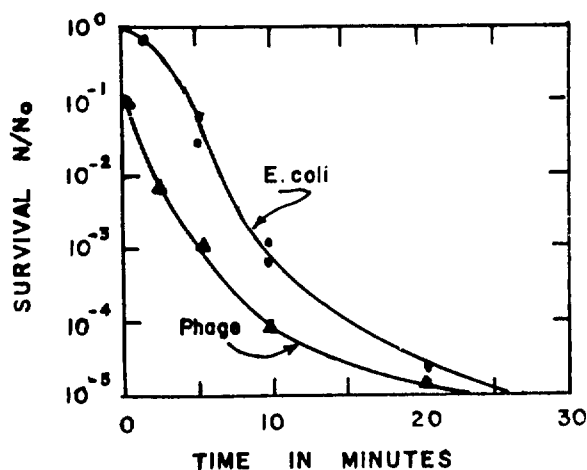


Figure 12. Effect of 4 ppm of bromine on phage and *E. coli* inactivation with varying time of contact. Temperature, 0°C; pH 7.5.

2. Effect of pH on the Activity of Bromine in Cells and Phage

Preliminary tests revealed that the inactivation of cells and phage at varying pH by 4 ppm bromine was so complete in 10 minutes that the true effect of pH could not be seen. Therefore, the concentration of bromine was lowered to 2 ppm with a contact time of 30 seconds at 0°C. The pH was varied from 3.8 to 5.6 with .05 M acetate buffer and from 5.85 to 8.05 with .05 M phosphate buffer. The results are presented in Figure 13 and show that the inactivation of phage and cells increased directly with the hydrogen ion concentration. The cells of *E. coli* appeared to be more resistant than the phage at all ranges of pH, but it was noted in previous tests that cells display about a 5 minute lag time for full reaction. This lag is not apparent with phage.

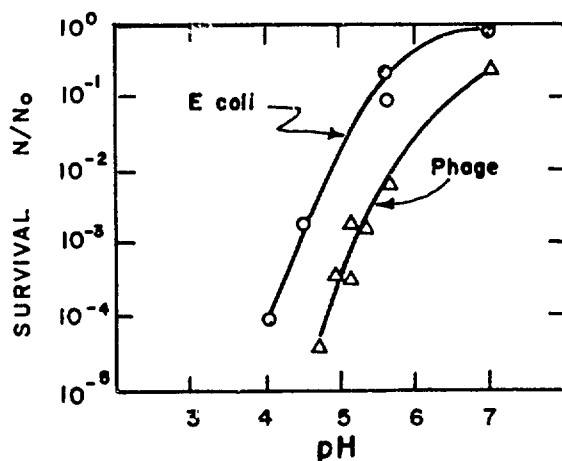


Figure 13. Effect of pH on inactivation of phage and *E. coli* by 2 mg/l bromine at 0°C for 30 seconds.

3. Effect of Br⁻ Concentration on Inactivation of Cells and Phage by

Bromine

The bromide ion was varied in the reaction of cells and phage with 4 ppm bromine at pH 7.5 and 0°C. Cell inactivation was stopped at 10 minutes. The phage kill, which appears to be more rapid, was terminated in 3 minutes. The tests were made in a range of bromide from 1×10^{-5} M to 1×10^{-1} M obtained by adding KBr. The results of these are presented in Figure 14. The bromide ion at a concentration of 10^{-1} M inhibited the cell inactivation to about two logs whereas phage inactivation was greatly enhanced. With no Br⁻ ion, in three minutes about 2 logs of phage kill would be expected, however, with 10^{-1} M KBr the kill was increased to about 6 logs. There is no explanation at this time for this unusual behavior.

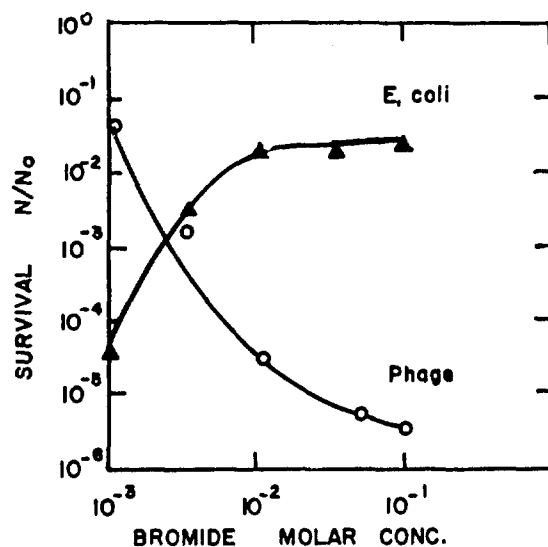


Figure 14. Effect of Br⁻ concentration on inactivation of phage and *E. coli* treated with 4 ppm bromine at pH 7.5, 0°C. Contact time, 10 minutes for cells, 3 minutes for phage.

4. Activity of RNA from Bromine Exposed f₂ Phage

Having previously demonstrated that purified DNA extracted from H. influenzae was quite sensitive to bromine, experiments were carried out with bromine treated phage.

Phage stock was added to a mixture of bromine at desired concentrations (0 to 15 ppm) in 5×10^{-3} M pH 7.5 phosphate buffer saline. After 10 minutes, the reaction was terminated by adding thiosulphate and the viability of the phage determined. RNA was extracted and tested for activity by the protoplast plating method already described.

The results are given in Figure 15 and show that intact phage and its extracted RNA were not equally sensitive to bromine. Whereas the intact phage was rapidly inactivated, the nucleic acid demonstrated a definite delay of about 3 logs of inactivation at each concentration of bromine.

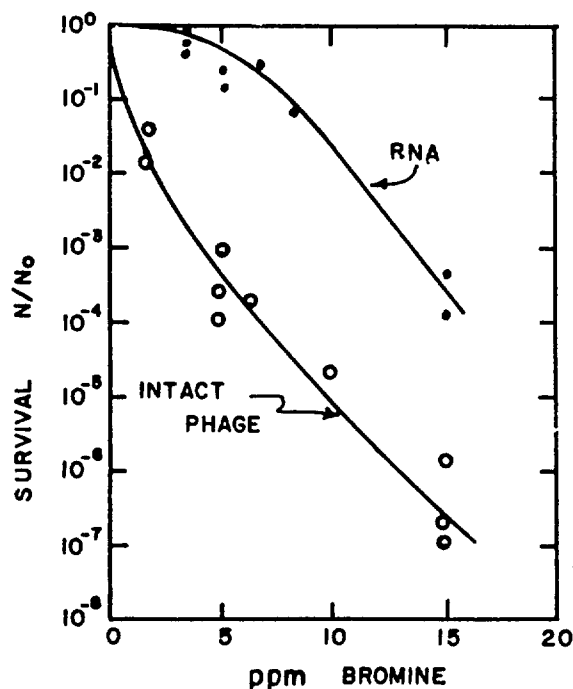


Figure 15. Activity of RNA extracted from bromine exposed f₂ phage. Temperature, 0°C; pH 7.5; reaction time 10 minutes.

DISCUSSION

In discussing the possible reaction of iodine with bacteria and viruses it is necessary to review briefly the current theory regarding the interaction of iodine with water. At the present time, the active species of iodine is regarded as the hydrated cationic iodine, H_2OI^+ (20,21,22) which attacks the base.

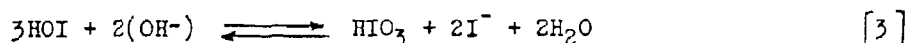
Research workers do not agree on just what species is most active in the disinfection process. In the past, most workers assume that I_2 or HOI , depending upon the pH, is the active form of iodine with little explanation how these uncharged species can react with the various proteins of amino acids involved. The fact that I_2 or HOI exists in overwhelming numbers of molecules compared to the hydrated cationic iodine tends to give support to the notion that these abundant forms are doing the work of disinfection.

The hydrolysis of elemental iodine in the formation of HOI , hypiodous acid, is shown in equation [1] and [2]



From these equilibrium equations it may be seen that the ratio $HOI:I_2$ co-exists at definite pH values and can be computed (23).

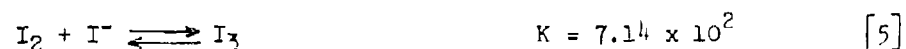
At an iodine dosage of 8 mg/l, diatomic I_2 will be the dominant species in the low pH range of water up to pH 7.0. Significant amounts of HOI exist in water beyond pH 7.0. The dissociation of HOI at high pH to give the hypiodite ion, OI^- , is not spectacular. Even at pH 10 the ratio of $HOI:OI^-$ is 220 to 1. As the hydroxyl ion concentration of the water is increased, the HOI is further oxidized as given below:



The active species of the hydrated cationic iodine is produced by the following reaction (20,22)



The actual number of molecules of H_2OI^+ produced is small, but so long as some I_2 is available and the concentration of I^- is minimal, the cationic form is generated as it is consumed in the reaction with organic materials. Equation [5] shows the combining of I_2 with the iodide to form the inactive tri-iodide.



From equations [4] and [5] the rate of reaction of iodine, as Hughes (20) has mentioned, is found to vary inversely as the square of the concentration of iodide, so that by increasing the I^- by even small amounts, the rate of iodine reaction will decrease rapidly. It should be recalled that the results on Figure 7 showed iodine inactivation varying as the cube of the iodide concentration.

The reaction of protein of amino acid with active species of iodine, H_2OI^+ , is shown in Figure 16. Among the three amino acids, the -SH group is the first one to be attacked by iodine, and the oxidation of the -SH group is not materially retarded by I^- ion.

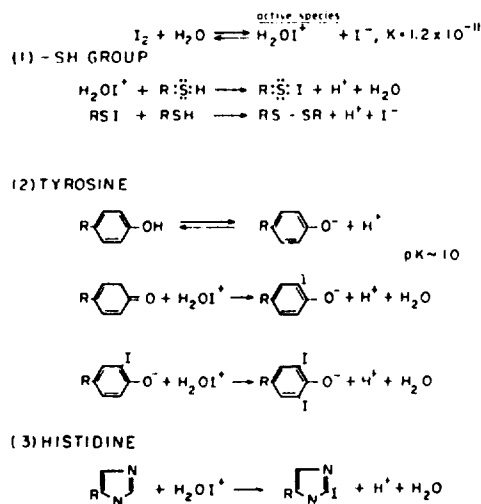


Figure 16. Reactions of the active species of iodine with (1) sulfhydryl group, (2) tyrosine, and (3) histidine proteins.

In addition, if the -SH group is the site of the inactivation, increasing the hydrogen ion (low pH) should favor inactivation. On the contrary, however, increasing the hydrogen ion (low pH) inhibits the iodination of tyrosine (24,19). It was reasoned, therefore, that the hydrolyzed cationic iodine, H_2OI^+ , will readily attack the phenolate ion of tyrosine ($R-C_6H_4-O^-$) or the quinoid form ($R-C_6H_4=O$) rather than the phenol form ($R-C_6H_4-OH$). Tyrosine in f_2 phage is the probable site of iodine inactivation and has been discussed elsewhere (25).

In the case of f_2 phage and poliovirus in which the tyrosine, but not the -SH groups, are the sites of iodine inactivation, lowering the pH would be undesirable. Thus, the difference of optimal pH which favors the inactivation of bacteria, but not viruses, might be explained by the difference in the inactivation sites.

Iodide ion is present in iodine solution, either intentionally as an iodine solubilizing agent, or unintentionally as a product of a reaction with organic matter. When the iodide ion concentration is sufficiently high (10^{-2} M) iodine inactivation of viruses ceases. This refers to an iodide concentration approaching 3000 ppm, a level which would not be attained under natural conditions. Most likely the inhibitory iodide ion formed as a product of the reaction of iodine on organic matter in the system was responsible for the report of inadequate poliovirus inactivation with a strong commercial iodine disinfectant (3). As for naturally occurring organic matter in water such as sewage, color and algae, the disinfection of virus may be retarded by the formation of the iodide ion.

Below is tabulated the 15 minute halogen demand of the effluent from a tertiary lagoon receiving completely treated domestic sewage. Varying dosages of chlorine and iodine have been added to compare the measured residuals of free and combined chlorine to that of free iodine. Iodoamines, if formed at all, are believed to be quite unstable.

COMPARISON OF CHLORINE AND IODINE DEMAND OF POND WATER
(pH, 7.7; 26°C; contact time, 15 minutes)

Halogen Added*	Chlorine Residual**	Iodine Residual**
ppm	ppm	ppm
10	4.3	0
20	11.1	0.2
30	19.8	4.1
40	27.3	6.8
50	36.5	14.9

*Chlorine stock made from chlorine gas
Iodine stock: $I_2:I^-$, 1 to 1.2

**By amperometric titration

Despite the low iodine residuals compared to those obtained with combined chlorine, a 20 ppm iodine dosage can be expected to provide about 2 to 3 logs of poliovirus inactivation under the above conditions. One characteristic of iodine as a disinfectant even in the presence of modest amounts of iodide is the rapidity at which inactivation takes place (see Figure 8).

Of greater concern is the inhibition of inactivation of viruses regardless of the presence of "free iodine" by low pH waters, high in organic matter and low in temperature.

SUMMARY

Since neither infectious RNA nor DNA is affected, iodine as a disinfectant probably inactivates bacteria and viruses by the iodination of the appropriate protein component.

The bactericidal action of iodine is complete within one minute of contact at 37°C. The rate of inactivation is not materially reduced by high concentrations of iodide ion. The destruction of bacteria continues until all iodine is consumed or, in case of residual iodine, until all bacteria are destroyed. The minimum number of iodine molecules required to destroy one bacterium varies with the species. For H. influenzae it was calculated to be 1.5×10^4 molecules of iodine per cell. When bacteria are treated with iodine, the inorganic phosphate up-take and oxygen consumption by the cells immediately ceases. Bacterial cells reacted with radioiodine show very little in the cell fraction, indicating that most likely an oxidation of -SH groups rather than a substitution into tyrosyl moieties occurs.

The inactivation of poliovirus and f₂ RNA bacteriophage by iodine is considerably inhibited by low levels of iodide ion concentration. At pH 6.0 or less iodine inactivation of f₂ phage was incomplete. The rate of inactivation by iodine in the presence of iodide ion for the f₂ virus and its host E. coli is different. The difference most likely is due to the nature of the iodine sensitive proteins involved.

The virucidal properties of iodine in waters of low pH and high organic content could be inadequate regardless of the free iodine concentration when virus or viruses involved require iodination of tyrosine for inactivation.

At the existing stage of the investigation on the mode of action of bromine on virus and cells, further work will be required to form definite conclusions. A general tentative summary is as follows:

- (1) Bacterial cells appear to be more resistant to bromine than the phage
- (2) Bromine activity on cells and phage varies directly with the hydrogen ion concentration and temperature.
- (3) The presence of the bromide ion enhances phage and inhibits cell inactivation by bromine.
- (4) The activity of RNA harvested from bromine treated phage is less affected than the intact phage.

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Mrs. Carole B. Anderson	1963-1966	technician
Mr. Karl E. Longley	1964-1965	graduate student
Miss Pauline Johnson	1963-1966	laboratory helper

Publications Resulting from this Research:

Hsu, Yu-Chih, Resistance of Infectious RNA and Transforming DNA to Iodine which Inactivates f₂ Phage and Cells. Nature vol. 203, No. 4941, pp. 152-153, July 11, 1964.

Hsu, Yu-Chih, Nomura, Shigeko, Krusé, Cornelius W. Some Bactericidal and Virucidal Properties of Iodine not Affecting Infectious RNA and DNA. Am. J. Epid., vol. 82, No. 3, pp. 317-328, Nov. 1965.